



Stephens, Francis B. and Chee, Carolyn and Wall, Benjamin T. and Murton, Andrew J. and Shannon, Chris E. and van Loon, Luc J.C. and Tsintzas, Kostas (2015) Lipid-induced insulin resistance is associated with an impaired skeletal muscle protein synthetic response to amino acid ingestion in healthy young men. *Diabetes*, 64 (5). pp. 1615-1620. ISSN 1939-327X

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**Lipid induced insulin resistance is associated with an impaired skeletal muscle protein
synthetic response to amino acid ingestion in healthy young men**

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Running title: Lipid induced anabolic resistance

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Word count: 1998

Tables: 0

Figures: 4

ABSTRACT

The ability to maintain skeletal muscle mass appears to be impaired in insulin resistant conditions that are characterised by muscle lipid accumulation, such as type 2 diabetes. The present study investigated the effect of acutely increasing lipid availability on muscle protein synthesis. Seven healthy young male volunteers underwent a 7 h intravenous infusion of L-[*ring*-²H₅]phenylalanine on two randomised occasions combined with either 0.9% saline or 10% Intralipid at 100 mL/h. After a 4 h ‘basal’ period, a 21 g bolus of amino acids was administered and a 3 h euglycaemic hyperinsulinemic clamp was commenced (‘fed’ period). Muscle biopsies were obtained from the vastus lateralis at 1.5, 4, and 7 h. Lipid infusion reduced fed whole-body glucose disposal by 20%. Furthermore, whereas mixed muscle fractional synthetic rate increased from the basal to fed period during saline infusion by 2.2-fold, no change occurred during lipid infusion, despite similar circulating insulin and leucine concentrations. This ‘anabolic resistance’ to insulin and amino acids with lipid infusion was associated with a complete suppression of muscle 4E-BP1 phosphorylation. We propose that increased muscle lipid availability may contribute to anabolic resistance in insulin resistant conditions by impairing translation initiation.

It has been proposed that the inability of skeletal muscle to adequately synthesise new protein in response to anabolic stimuli such as amino acids (termed ‘anabolic resistance’) is a key contributory factor to the muscle mass loss observed in a variety of conditions such as ageing, type 2 diabetes, disuse, and critical illness (e.g. 1, 2). A common feature of all these conditions is the inability of skeletal muscle glucose metabolism to respond adequately to insulin signalling (insulin resistance), which is thought to be a consequence of the intracellular accumulation of lipid within skeletal muscle (3). As insulin signalling is also integral to skeletal muscle amino acid delivery and metabolism, in particular playing a permissive role in regulating muscle protein synthesis via activation of the mammalian target of rapamycin complex 1 (mTOR) pathway (4, 5), it is possible that lipid-induced insulin resistance may contribute towards skeletal muscle anabolic resistance. Indeed, skeletal muscle protein synthesis in response to insulin and amino acids appears to be negatively related to whole body fat mass and insulin sensitivity in humans (6). Thus, it is important to elucidate the impact of excess lipid *per se* on insulin and amino acid stimulated skeletal muscle protein synthesis and the associated signalling pathways *in vivo* in humans, particularly if strategies to treat anabolic resistance are to focus on reducing skeletal muscle lipid accumulation and insulin resistance.

The intravenous infusion of a lipid emulsion with heparin is routinely used to elevate free fatty acid availability and allows researchers to investigate the acute effects of lipid-induced insulin resistance on insulin signalling (7) and subsequent impairments in glucose uptake, storage and oxidation (8, 9). The present study investigated the impact of acutely elevating fatty acid availability to a concentration known to induce insulin resistance of glucose metabolism on the muscle protein synthetic response and associated signalling to amino acid ingestion in the presence of a controlled, steady-state circulating insulin concentration in humans.

RESEARCH DESIGN & METHODS

Subjects

Seven healthy male participants (age 23.0 ± 0.8 years, body mass 78.5 ± 3.8 kg, BMI 24.5 ± 0.9 kg/m²) gave their written informed consent to participate in the present study, which was approved by the University of Nottingham Medical School Ethics Committee in accordance with the Declaration of Helsinki.

Protocol

Participants reported to the laboratory at 0800 on two randomized occasions, at least two weeks apart, following an overnight fast and having abstained from strenuous exercise for the previous 48 hours. On each visit the participants rested semi-supine on a bed whilst a 7 h intravenous infusion of L-[*ring*-²H₅]phenylalanine ($0.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$; Cambridge Isotopes Limited, MA, USA) was performed in combination with the infusion of 0.9% saline ('Control') or 10% Intralipid ('Lipid'; Fresenius Kabi, Germany) at a rate of 100 ml/h. During the lipid infusion heparin was infused at rate of 600 U/h to elevate plasma non-esterified fatty acid (NEFA) availability. After 4 h a 21 g bolus of amino acids (except phenylalanine and tyrosine; Tyrosidon, SHS International Ltd., UK) was administered in a 440 mL solution via a nasogastric tube to avoid issues with palatability. At the same time a 3-hour hyperinsulinemic (Actrapid, Novo Nordisk, Denmark) euglycaemic (20% dextrose, Baxter Healthcare, UK) clamp (10) was commenced at a rate of $50 \text{ mU} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$. At $t = 7$ h, the Intralipid/saline, L-[*ring*-²H₅]phenylalanine, and insulin infusions were stopped, whereas the glucose infusion was continued until blood glucose concentration was stable.

Sample collection and analysis

Arterialized-venous blood was obtained from a heated hand vein (11) at $t = 0$, 1.5 h and every 30 min thereafter. Plasma treated with tetrahydrolipostatin (30 µg/ml plasma) was analysed for NEFA (NEFA C kit, WAKO Chemicals, Germany) on an automated analyzer (ABX

Pentra 400, Horiba Medical Ltd., France). Plasma separated from EGTA treated blood was analysed for insulin concentration by ELISA (DRG diagnostics, Germany) and, after deproteinisation on ice with dry 5-sulfosalicylic acid, phenylalanine and leucine concentration, and L-[ring- $^2\text{H}_5$]phenylalanine enrichment by GC-MS (Agilent 7890A GC/5975C; MSD, Little Falls, USA) as previously described (12, 13).

Muscle samples were obtained from the *vastus lateralis* at $t = 1, 4.5$, and 7 h using the Bergström needle biopsy technique, and immediately frozen in liquid nitrogen. Freeze-dried muscle separated free of visible blood, fat, and connective tissue was analysed for intracellular tissue free and protein-bound L-[ring- $^2\text{H}_5$]phenylalanine enrichments (12, 13), as well as acetylcarnitine and long-chain acyl-CoA ($t = 1$ and 7 h only) as previously described (9). The remaining 'wet' muscle was used to measure the total muscle protein content ($t = 4.5$ and 7 h only) of phosphorylated Akt (serine 473), mTOR (serine 2448), and 4E-BP1 (threonine $^{37/46}$) by Western blot analysis using commercial antibodies (Cell Signalling, MA, USA) normalised to α -actin (Sigma-Aldrich Company Ltd., UK) to control for loading, as well as pyruvate dehydrogenase complex activation status (PDCa), both as described previously (9).

Calculations

Fractional rate of mixed muscle protein synthesis (FSR) was calculated by dividing the increment in enrichment in the product (i.e. protein-bound L-[ring- $^2\text{H}_5$]phenylalanine) by the enrichment of the precursor (i.e. plasma free L-[ring- $^2\text{H}_5$]phenylalanine; 13). To adjust for non-steady state plasma tracer enrichments during the fed period, precursor enrichments were calculated as the integral of the plasma L-[ring- $^2\text{H}_5$]phenylalanine enrichment (14, 15)

Statistics

A two-way ANOVA (time and treatment factors; GraphPad Prism 6, GraphPad Software Inc, USA) was performed to detect differences within and between groups for all measures

described. When a significant effect was observed, a Student's t-test with Bonferroni correction was performed to locate differences. Statistical significance was declared at $P < 0.05$. All the values presented in text, Tables and Figures represent mean \pm the standard error of the mean.

RESULTS

Insulin resistance of glucose metabolism

Steady-state plasma NEFA concentrations were elevated throughout the basal period in Lipid compared to Control ($P < 0.001$; Figure 1A). Furthermore, plasma NEFA concentrations were suppressed ($P < 0.001$) by insulin and amino acid administration to a similar degree (-0.38 ± 0.01 vs. -0.39 ± 0.01 mmol/l; Figure 1A) such that they remained greater in Lipid throughout the fed period ($P < 0.001$; Figure 1A). This similar suppression of plasma NEFA concentration between Lipid and Control was reflected by similar-steady state blood glucose (4.51 ± 0.05 vs. 4.52 ± 0.05 mmol/l, respectively) and plasma insulin concentrations (104 ± 5 vs. 99 ± 3 mU/l, respectively; Figure 1B). However, there was a $20 \pm 6\%$ lower average glucose disposal during the final hour of the fed period in Lipid compared to Control ($P < 0.01$; Figure 1C) and a $56 \pm 12\%$ lower PDCa by the end of the fed period at 7 h ($P < 0.05$; Figure 1D).

Amino acid and lipid metabolism

Plasma leucine and phenylalanine concentrations (Figure 2A and 2B, respectively) were both maintained at fasting concentrations throughout the basal period in Lipid and Control. Similarly, plasma L-[ring- $^2\text{H}_5$]phenylalanine enrichments remained at the same steady-state levels in Lipid and Control (Figure 2C). Insulin and amino acid administration resulted in a similar peak in plasma leucine concentration in Lipid and Control after 30 min of the fed period ($P < 0.001$; Figure 2A). However, insulin and amino acid administration caused a steady decline in plasma phenylalanine concentration during Control such that it was $49 \pm 3\%$ lower during the final hour of the fed period compared to basal ($P < 0.001$; Figure 2B). Furthermore, the degree of reduction in plasma phenylalanine concentration in response to insulin and amino acid administration was greater in Lipid ($65 \pm 3\%$; $P < 0.001$) when compared with Control, such that steady-state phenylalanine concentrations were lower ($P < 0.001$; Figure 2B). However, this did not result in a greater L-[ring- $^2\text{H}_5$]phenylalanine enrichment during the final hour of the fed period in Lipid compared to Control (Figure 2C).

Basal mixed muscle FSR was the same in Lipid compared to Control (Figure 3A). However, whereas mixed muscle FSR increased from the basal to fed period in Control ($P<0.05$; Figure 3A), it did not respond to insulin and amino acid administration in Lipid, such that it was significantly lower than Control ($P<0.05$; Figure 3A). This was despite similar intracellular muscle enrichment before and after insulin and amino acid administration (4.4 ± 0.3 to 6.0 ± 0.3 MPE vs. 4.6 ± 0.3 to 5.9 ± 0.6 MPE for Lipid and Control, respectively). Furthermore, this lower rate of protein synthesis in Lipid coincided with an inhibition of the insulin- and amino acid-mediated decline in skeletal muscle long-chain acyl-CoA ($P<0.05$; Figure 3B) and acetylcarnitine ($P<0.01$; Figure 3C) content at $t = 7$ h in Control.

Associated muscle signalling pathways

The phosphorylation status of Akt, mTOR, and 4E-BP1 were the same after the basal period at $t = 4$ h in Lipid compared to Control. Insulin and amino acid administration increased the phosphorylation of Akt ($P<0.05$; Figure 4A), mTOR ($P<0.01$; Figure 4B), and 4E-BP1 ($P<0.001$; Figure 4C) by 1.9-, 1.7-, and 2.9-fold, respectively, compared to basal in Control. However, whereas insulin and amino acid administration also increased mTOR phosphorylation by 1.5-fold from basal in Lipid ($P<0.05$; Figure 4B), the 1.8-fold increase in Akt phosphorylation was not significantly different ($P=0.09$; Figure 4A). Furthermore, Lipid infusion had no effect on 4E-BP1 phosphorylation at all such that it was less than half that of Control at the end of the fed period ($P<0.01$; Figure 4C).

DISCUSSION

The concept that lipid-induced insulin resistance coincides with anabolic resistance is not new. Diet-induced obesity in mice (16) and rats (17) was shown to impair the activation of skeletal muscle protein synthesis in response to feeding, particularly in glycolytic muscle where there was chronic lipid infiltration (17). The whole body protein anabolic response to combined hyperinsulinaemia and hyperaminoacidaemia is also blunted in obese women when compared to lean individuals (18), and skeletal muscle protein synthesis in response to insulin and amino acids appears to be negatively related to whole body fat mass in humans (6). However, the contribution of excess lipid and insulin resistance to anabolic resistance cannot be determined from the associations described above as other confounding factors, particularly physical activity status, will also contribute to anabolic resistance (2, 19). Thus, the present study clearly demonstrates that excess lipid availability *per se* within skeletal muscle can induce both insulin resistance of skeletal muscle glucose metabolism and anabolic resistance of amino acid metabolism, independently of physical activity levels and diet-induced changes in body composition. This was evidenced by reduced insulin-stimulated peripheral glucose disposal and skeletal muscle PDC activation by around 20 and 50%, respectively, and a complete prevention of a 2.2-fold increase in the rate of mixed muscle protein synthesis in response to the ingestion of 21 g of amino acids containing 2.3 g leucine. Furthermore, this inability of skeletal muscle to increase protein synthesis in response to insulin and amino acid administration appeared to be mediated in part via the repression of translation initiation at the level of 4E-BP1, which is in agreement with previous studies demonstrating that elevating lipid availability in rats and cells decreases muscle protein synthesis (20, 21).

This is, to our knowledge, the first study to acutely induce anabolic resistance in humans via lipid administration. However, these findings are in contrast to the study of Katsanos et al (22), where lipid was intravenously administered at nearly twice the rate and there was no blunting of the 50% increase in muscle protein synthesis observed in response to the ingestion

of 7 g of essential amino acids. The differences are difficult to reconcile, but may be due to the hyperinsulinaemic euglycaemic clamp in the present study, which likely impaired fat oxidation and caused an accumulation of intracellular lipid metabolites (23). Indeed, this was reflected by greater muscle long-chain acyl-CoA and acetylcarnitine content during lipid infusion under insulin and amino acid stimulated conditions, which are sensitive markers of incomplete lipid oxidation and lipid accumulation (9, 24). Whilst a mechanistic link has not been established, these results offer compelling evidence that a common lipid-mediated intracellular signalling defect downstream of the Akt-mTOR signalling pathway may cause both lipid-induced insulin and anabolic resistance, and provide insight into how conditions of lipid-induced insulin resistance such as ageing, type 2 diabetes, disuse, and critical illness may be linked to accelerated muscle mass loss.

ACKNOWLEDGEMENTS

Author contributions: FBS wrote the manuscript and takes responsibility for its content. FBS, BTW, LJCvL, and KT designed the study. All of the coauthors researched data and reviewed and edited the manuscript.

This research study was funded by the University of Nottingham and Maastricht University. AJM was supported by a British Nutrition Foundation Drummond Pump Priming Award. No potential conflicts of interest relevant to this article were reported. We would like to thank Ian Bennett (University of Nottingham) and Joan Senden (Maastricht University) for technical assistance. Abstracts of this study (W217 and LB813) were presented at Experimental Biology 2014, San Diego, USA.

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FIGURE LEGENDS

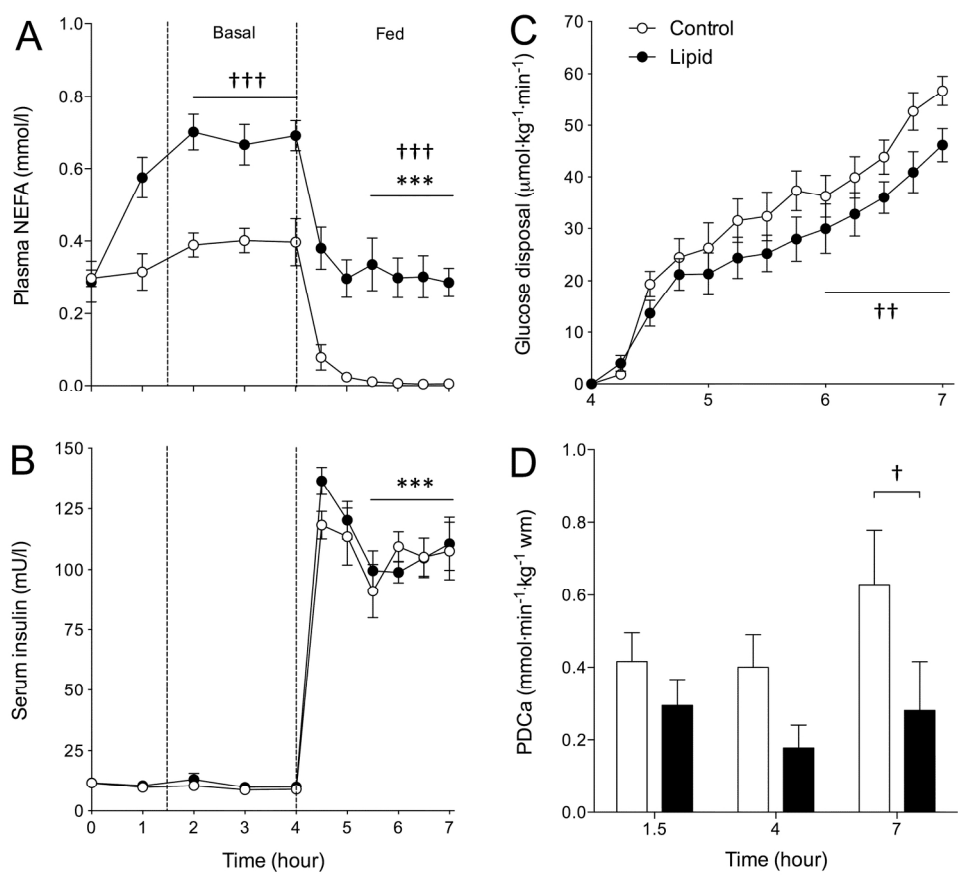
Figure 1. Plasma NEFA concentration (A), plasma insulin concentration (B), whole-body glucose disposal (C), and PDCa (D) before (Basal 1.5-4 h) and after (Fed 4-7 h) the administration of 21 g of amino acids and a 3 h euglycaemic hyperinsulinaemic (~100 mU/l) clamp during 7 h intravenous infusion of saline (Control; white circles) or 10% Intralipid (Lipid; black circles) at a rate of 100 ml/h. Values represent means \pm SEM. $^{\dagger\dagger\dagger} P<0.001$, $^{\dagger\dagger} P<0.01$, $^{\dagger} P<0.05$, Lipid significantly different from corresponding Control value. $^{***} P<0.001$, Control and Lipid during Fed significantly different from corresponding Basal steady-state.

Figure 2. Plasma leucine concentration (A), plasma phenylalanine concentration (B), and plasma phenylalanine enrichment (C), before (Basal 1.5-4 h) and after (Fed 4-7 h) the administration of 21 g of amino acids and a 3 h euglycaemic hyperinsulinaemic (~100 mU/l) clamp during 7 h intravenous infusion of saline (Control; white circles) or 10% Intralipid (Lipid; black circles) at a rate of 100 ml/h. Values represent means \pm SEM. $^{\dagger\dagger\dagger} P<0.001$, Lipid significantly lower corresponding Control value. $^{***} P<0.001$, $^{**} P<0.01$, Control and Lipid during Fed significantly different from corresponding Basal values.

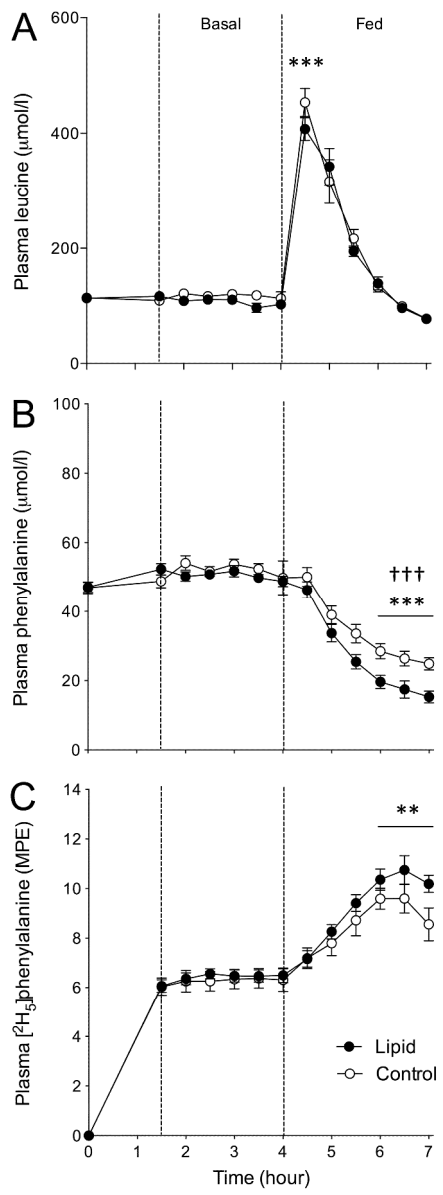
Figure 3. Skeletal muscle mixed protein fraction synthetic rate (A), long-chain acyl-CoA content (B) and acetylcarnitine content (C) before (Basal) and after (Fed) the administration of 21 g of amino acids and a 3 h euglycaemic hyperinsulinaemic (~100 mU/l) clamp during 7 h intravenous infusion of saline (Control; white bars) or 10% Intralipid (Lipid; black bars) at a rate of 100 ml/h. Values represent means \pm SEM. $^{\dagger} P<0.05$, $^{\dagger\dagger} P<0.01$, Lipid significantly lower than corresponding Control value. $^{*} P<0.05$, $^{***} P<0.001$, Fed significantly greater from corresponding Basal values.

Figure 4. Representative blots (top panel) and phosphorylation status of skeletal muscle Akt

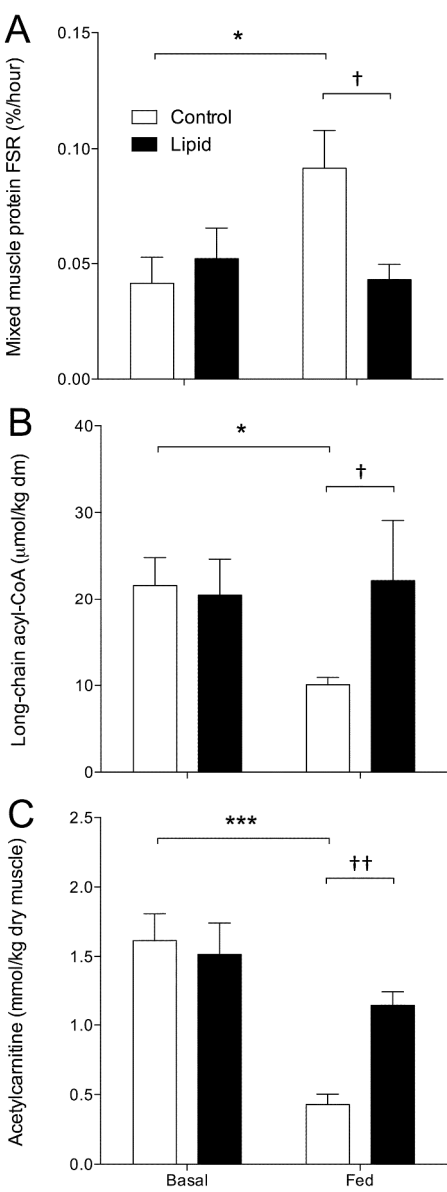
serine⁴⁷³ (A), mTOR serine²⁴⁴⁸ (B), and 4E-BP1 threonine^{37/46} (C) before (Basal) and after (Fed) the administration of 21 g of amino acids and a 3 h euglycaemic hyperinsulinaemic (~100 mU/l) clamp during 7 h intravenous infusion of saline (Control; white bars) or 10% Intralipid (Lipid; black bars) at a rate of 100 ml/h. Values represent means \pm SEM. ^{††} $P < 0.01$, Lipid significantly lower than corresponding Control value. ^{***} $P < 0.001$, ^{**} $P < 0.01$, ^{*} $P < 0.05$, Fed significantly greater from corresponding Basal values.



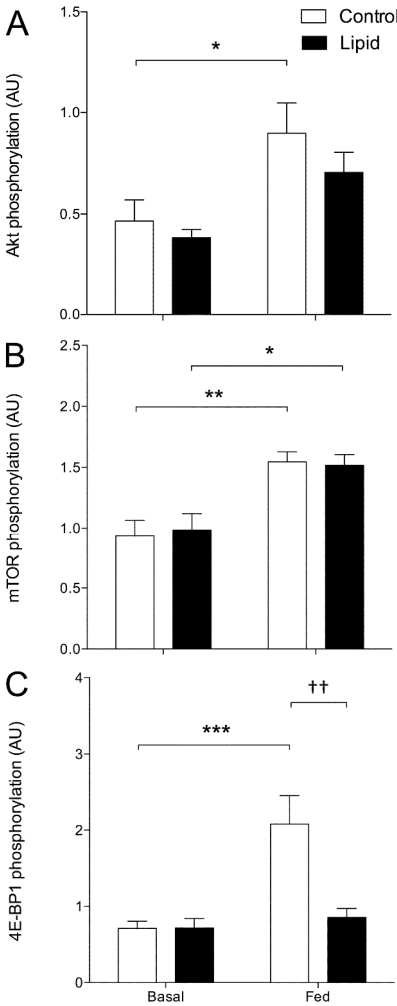
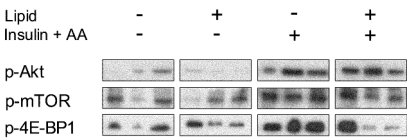
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264x665mm (300 x 300 DPI)



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